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Communication

Macrophages Adhere to Glucose-modified Basement Membrane Collagen IV via Their Scavenger Receptors*

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Scavenger receptors have been reported to mediate macrophage adhesion to serum-coated plastic surfaces. We report here that scavenger receptors promote the divalent cation independent adhesion of human monocytes and macrophages to surfaces coated with non-enzymatically glycated collagen IV but not to surfaces coated with native collagen IV. Ligands for scavenger receptor types I and II blocked adhesion of monocytes and macrophages to non-enzymatically glycated collagen IV but had no effect on adhesion of these cells to albumin-coated surfaces. U937 human promonocyte-like cells transfected with cDNA encoding bovine scavenger receptor I or II adhered to surfaces coated with glycated-collagen IV but not to surfaces coated with native collagen IV. A synthetic peptide homologous to the domain of bovine scavenger receptor that binds modified low density lipoproteins (residues 327–343) inhibited the adhesion of U937 cells transfected with cDNA encoding bovine scavenger receptor II to glycated collagen IV, whereas a control peptide from the α helical domain of scavenger receptor II (residues 121–137) had no effect on adhesion of these cells. Macrophages plated on surfaces coated with glycated collagen IV were unable to endocytose acetylated low density lipoproteins from the medium, suggesting that their scavenger receptors were occupied in binding these cells to the substrate. These findings suggest new roles for scavenger receptors in the accelerated development of vascular lesions observed in diabetics.

Mononuclear phagocytes express scavenger receptors (SRs)[1] that promote endocytosis and degradation of modified low density lipoproteins (1). Two types of SRs have been identified and cloned (2, 3). Both types are homotrimeric membrane glyco-proteins with similar ligand binding properties. SR type II differs from SR type I by the presence of a cysteine-rich domain in the extracellular carboxyl terminus of SR I (3). SRs bind a wide variety of ligands including proteins, polynucleotides, and sulfated polysaccharides (4). Recently, SRs have been shown to promote adhesion of murine macrophages to serum-coated plastic surfaces (5). The protein ligands for SRs all share a common characteristic of having their lysine residues modified (6), rendering the proteins more negatively charged.

Non-enzymatic glycation of arterial basement membrane proteins occurs in aging and occurs at an accelerated rate in diabetics. With time, these glucose adducts rearrange chemically to form advanced glycation end products (7). Since non-enzymatic glycation modifies lysine residues of proteins (8, 9), we examined whether SRs recognize non-enzymatically glycated proteins. The studies reported here show that macrophages interact with surfaces coated with non-enzymatically glycated basement membrane collagen type IV via their SRs, and that these interactions block the capacity of macrophages adherent to surfaces coated with glycated collagen IV to endocytose AcLDL in the medium.

EXPERIMENTAL PROCEDURES

Materials—Bovine collagen IV was obtained from ICN (Irvine, CA); glucose was from Fluka Biochem (Ronkonkoma, NY); low density lipoproteins (LDL), AcLDL, and DiI-labeled native (DiI-LDL), and acetylated (DiI-AcLDL), were obtained from the Cardiovascular Research Institute (Organon Teknika, Durham, NC). Competent MC1061/p3 *Escherichia coli* and the mammalian expression vector PcDNA1neo were from Invitrogen (San Diego, CA). The mammalian expression vectors PxSR3 and PxSR7 for bovine type II and type I scavenger receptors, respectively, were a generous gift from Dr. Monty Krieger (Massachusetts Institute of Technology). The human promonocytic cell line U937 (10) was obtained from ATCC and was maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Upstate Biotechnology, Inc., Lake Placid, NY), penicillin (100 units/ml), and streptomycin (100 µg/ml) (medium A). Poly-L-lysine (M, 5200) was from Sigma.

Plasmids and Transfections—To produce U937 cells expressing type II SRs, PxSR3 was digested with BamHI and XhoI and the resulting cDNA fragment encoding the type II bovine SR was ligated into the appropriate sites of PcDNA1neo, generating a plasmid "PxSR3neo." Unstimulated U937 cells (10⁷) were transfected with 5 µg PxSR3neo by electroporation. To generate U937 cells expressing the type I bovine SR, 10⁷ U937 cells were cotransfected with 10 µg of PxSR7 and 2 µg of PcDNA1neo. Transfectants were cultured in 24-well plates in medium A supplemented with 670 µg/ml G418 (Life Technologies, Inc.). Individual G418-resistant U937 clones were harvested and analyzed for uptake of AcLDL. Cells were incubated in RPMI 1640 containing 10 µg/ml fluorescent DiI-AcLDL and 10% delipidized fetal bovine serum for 6 h at 37 °C. Cell-associated DiI-AcLDL was quantitated by fluorescence-activated cell sorting (FACS) analysis. Several U937 clones that took up DiI-AcLDL (i.e. expressing SR) were identified in this manner. U937 cells that were transfected with the vector PcDNA1neo alone showed undetectable uptake of DiI-AcLDL by FACS. These cells were used as controls in all experiments.

Monocyte Isolation and Culture—Human monocytes were isolated from leukocyte concentrates (New York Blood Center, New York, NY) by centrifugation over Ficoll Hypaque (Sigma) as described (11). The mononuclear cell layer was collected, washed three times in RPMI 1640, and resuspended in the same medium supplemented with 20% pooled human serum (Gemini, Calabasas, CA). The cells were allowed to adhere to tissue culture plates for 1 h in the same medium at 37 °C. Nonadherent cells were washed away; adherent cells were detached by a brief incubation with phosphate-buffered saline containing 5 mM EDTA at 4 °C followed by tapping the side of the dish as described (11). This method yielded >90% monocytes as evidenced by nonspecific esterase

FIG. 1. **Increased fluorescence of CIV incubated with glucose.** 1 mg ml bovine CIV was incubated in PD buffer with 0 mM (●), 50 mM (■), or 500 mM (▲) of glucose for up to 8 weeks as described under "Experimental Procedures." At 2-week intervals, aliquots were taken and fluorescence was quantitated using a 650-40 Perkin Elmer fluorescence spectrophotometer (excitation 370, emission 440).

staining and by the ability of the recovered cells to phagocytose IgG-coated sheep red blood cells. To obtain monocyte-derived macrophages, monocytes separated as above were cultured for 7 days in RPMI 1640 supplemented with 30% pooled human serum in Teflon beakers, as described [12].

Protein Glycation—Collagen IV was glycated as described [13-15, 19]. Briefly, collagen IV (1 mg/ml) in phosphate-buffered saline without Ca^{2+} or Mg^{2+} (PD buffer), with or without 500 mM glucose, was sterilized by filtration through a 0.2-µm filter (Gelman Sciences, Ann Arbor, MI) and incubated for the indicated periods of time at 37 °C in the dark in a tightly closed tube. 1 ml of this mixture was dialyzed three times against 500 ml of PD buffer at 4 °C for 12 h. Collagen IV treated in this way showed no evidence of degradation as evidenced by gel electrophoresis, and contained advanced glycation end products as measured by increased fluorescence compared to native collagen IV (Refs. 13 and 14 and Fig. 1). The extent of glycation was proportional to the length of time the collagen was incubated with glucose and the glucose concentration used (Fig. 1).

Adhesion Assay—10 µl of deionized H_2O containing 10 µg of native or glycated collagen IV or human serum albumin (HSA) was placed on each spot of a Multispot glass slide (6 mm diameter of spots) (Shandon, Pittsburgh, PA). The slides were air-dried in a laminar flow tissue culture hood. 50 µl of serum-free RPMI 1640 supplemented with 1 mg ml BSA and containing 50,000 cells was placed on each spot, and the slides were placed in a CO_2 incubator at 37 °C for 1 h to allow cells to adhere. The slides then were washed three times with serum-free RPMI, and the number of cells adherent to each spot was determined by one of two methods. 1) Slides were fixed in 3.7% formaldehyde, and the number of cells in a 1-mm² grid was counted by light microscopy. 2) Slides were incubated for 15 min at 37 °C in RPMI containing 0.33 µg ml neutral red (16) (Sigma) and washed three times with RPMI at room temperature. 50 µl of an aqueous solution containing 50% methanol, 1% acetic acid was added to each spot and the optical density of each spot was measured in a microplate reader at 540 nM. Standard curves were generated by allowing varying numbers of cells to adhere to the glass slides and then processing them as described above. We generated a standard curve for each neutral red assay. Control experiments showed that the neutral red assay accurately measured the number of viable cells in each spot and correlated well with measurements of the number of cells adherent to the slides. Fc receptor-mediated phagocytosis was assayed as described (17).

Synthetic Peptides—The synthetic peptide KPGLNGQKGQKGEK-GSS SP1 corresponding to the collagenous-like domain (3) and a contiguous peptide ESRIQYLSDNEANLLDA (SP2) from the α helical domain of bovine SRII (3) were synthesized by Chiron Mimotopes U.S. (Raleigh, NC) and used in the adhesion studies as described below.

RESULTS AND DISCUSSION

Human monocytes and monocyte-derived macrophages adhered poorly to native collagen IV (Fig. 2a and Ref. 18). In contrast, both monocytes and macrophages adhered avidly to glycated collagen IV (G-CIV) (Fig. 2a). Twice as many macrophages adhered to G-CIV as did monocytes (Fig. 2a). To test whether macrophages adhere to G-CIV via their scavenger receptors we plated these cells on surfaces coated with G-CIV in medium containing ligands of SRs (4) (i.e., acetylated LDL, AcLDL, fucoidan, polyinosinic acid (poly(I)), and polyguanilic acid (poly(G)), or in medium containing polycytidylic acid
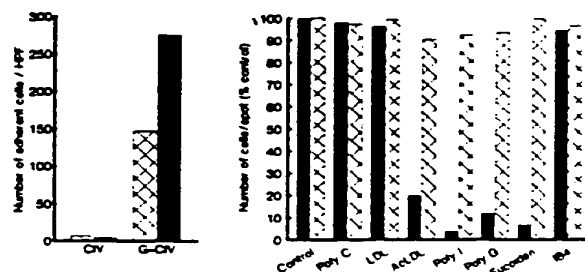
FIG. 2. *a*, monocytes and macrophages adhere to G-CIV but not to native CIV. Multispot slides were coated with 10 µg of native or glycated collagen IV per spot. 5 x 10⁴ monocytes (□) (1 day in culture) or 5 x 10⁴ monocyte-derived macrophages (■) (7 days in culture) were allowed to adhere to each spot. The slides were processed as described under "Experimental Procedures," and the number of cells per high power field was counted. (The distribution of cells over the substrate was uniform.) Each value is the average of 10 randomly picked high power fields. The experiment was repeated three times giving similar results each time. The results reported are for a representative experiment. *b*, SR ligands block adhesion of macrophages to G-CIV. 50 µl of RPMI medium containing 5 x 10⁴ monocyte-derived macrophages (7 days in culture), and 25 µg/ml of either poly(I), poly(G), poly(C), or monoclonal antibody IB4, or 100 µg/ml of either LDL, AcLDL, or fucoidan, was placed on each spot of albumin-coated (□) or glycated collagen IV-coated (■) Multispot slides. The slides were incubated for 1 h at 37 °C and washed with RPMI, and the number of adherent cells was quantitated by the neutral red assay as described under "Experimental Procedures" (100% control value for G-CIV-coated slides = 30 x 10⁴ cells/spot; for albumin-coated slides = 36 x 10⁴ cells/spot). The experiment was repeated eight times, each time in triplicate, with similar results. The results shown are for a representative experiment.

(poly(C)) or LDL. AcLDL, fucoidan, poly(I), and poly(G) each inhibited the adhesion of macrophages to glycated collagen IV-coated surfaces, whereas poly(C) and LDL did not (Fig. 2b). Control experiments showed that AcLDL, fucoidan, poly(I), and poly(G) did not affect the adhesion of macrophages to albumin-coated glass (Fig. 2b).

The Mac-1 and p150,95 β_2 integrins mediate the binding of leukocytes to a variety of extracellular matrix proteins (20, 21), as well as to heat and urea denatured proteins in a divalent cation-dependent manner (21). To test whether β_2 integrins are involved in the adhesion of macrophages to G-CIV, we added 5 mM EDTA to the adhesion medium to chelate divalent cations. Addition of 5 mM EDTA had no effect on the adhesion of macrophages to G-CIV (not shown), indicating that the binding is divalent cation-independent. This is consistent with previous reports that binding of ligands to SRs is cation-independent (1, 5) and suggests that β_2 integrins are not involved. Indirect immunofluorescence studies confirmed that monoclonal antibody IB4 directed against β_2 integrins on human leukocytes (23), bound to macrophages. As expected, however, IB4 had no effect on adhesion of macrophages to G-CIV (Fig. 2b). Control experiments showed that IB4 was active, since it blocked adhesion of human polymorphonuclear leukocytes and macrophages to fibrinogen-coated substrates (data not shown) as described (20).

Unstimulated U937 cells do not express scavenger receptors (22), and do not adhere to surfaces coated with native or with G-CIV (not shown). To confirm that SRs play a role in adhesion of mononuclear phagocytes to G-CIV, we isolated several clones of U937 cells stably transfected with a plasmid encoding bovine type I (SRIA6 cells) or type II (SRIIA4 cells) SR or with the vector pcDNA1neo (VB3 cells). Fluorescence microscopy and flow cytometric analysis of the uptake of DiI-AcLDL (a known ligand for SRs) by SRIIA4, SRIA6, and VB3 cells showed that SRIA6 and SRIIA4 cells bound and endocytosed 7 and 7.5 times, respectively, more DiI-AcLDL than VB3 cells (Fig. 3a
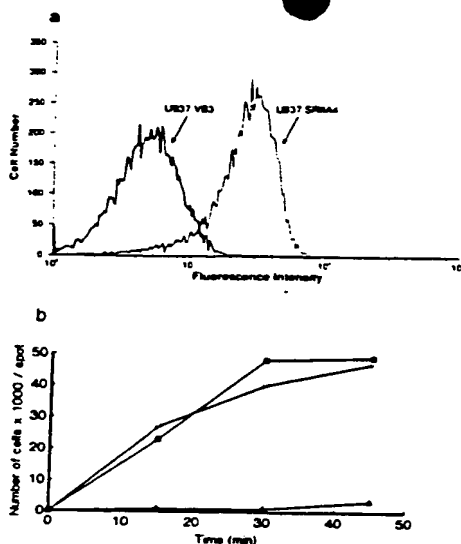
Fig. 4. **Ligands of SRs block adhesion of SR-transfected U937 to G-CIV.** 50 µl of RPMI containing 1 mg/ml BSA, 5 × 10⁴ SRIIA4 cells, and 25 µg/ml of either poly(I), poly(G), or poly(C), or 100 µg/ml of either LDL, AcLDL, or fucoidan, or 10 µg/ml monoclonal antibody IB4 was placed on each spot of Multispot slides that were coated with glycated collagen IV (G-CIV) (■) or with albumin-coated (□) spots, and the cells were incubated, processed, and counted exactly as described in Fig. 2 legend. The experiment was repeated three times with similar results. The values reported here are the average of four determinations from a representative experiment (control values for G-CIV-coated slides = 45 × 10³ cells/spot and for albumin-coated slides = 48 × 10³ cells/spot.

Fig. 3. *a*, uptake of DiI AcLDL by U937 cells transfected with SRII. 5 × 10⁶ SRIIA4 cells (U937 cells transfected with bovine scavenger receptor type II), or 5 × 10⁶ VB3 cells (U937 cells transfected with vector alone), were incubated in 5 ml of RPMI containing 10 µg/ml DiI-labeled AcLDL for 6 h at 37°c. The cells were washed three times in RPMI, and cell-associated fluorescence was quantitated by FACS analysis. Unlabeled AcLDL totally inhibited the uptake of DiI AcLDL by SRIIA4 cells (not shown). Similar results were obtained with SRIA6 cells (U937 cells transfected with bovine scavenger receptor type I)(not shown). *b*, U937 cells transfected with SRI and SRII adhere to G-CIV. 5 × 10⁶ SRIIA4 (■), SRIA6 (○), or VB3 (▲) cells in RPMI containing 1 mg/ml BSA were added at 37 °C to each spot of a Multispot slide. Each spot was coated with 10 µg of glycated collagen IV as described under "Experimental Procedures." Values reported are the average of six determinations in a representative experiment. The experiment was repeated 16 different times for SRIIA4 and VB3 and three times for SRIA6 over a period of 6 months using different lots of glycated collagen IV with similar results.

and data not shown). SRIA6, SRIIA4, and VB3 cells were incubated with surfaces coated with either native or G-CIV. Over 90% of SRIA6 and SRIIA4 cells adhered to surfaces coated with G-CIV. In contrast, fewer than 5% of VB3 cells adhered to surfaces coated with G-CIV (Fig. 3*b*), and fewer than 5% of SRIA6, SRIIA4, or VB3 cells adhered to surfaces coated with native collagen IV (not shown).

To further verify that binding of SRIA6 and SRIIA4 cells to G-CIV is mediated by SRs, we suspended these cells in medium containing AcLDL, LDL, fucoidan, poly(I), poly(G), or poly(C) and plated them at 37 °C on surfaces coated with G-CIV (Fig. 4). Only ligands for SRs (AcLDL, fucoidan, poly(I), and poly(G)) blocked adhesion of SRIIA4 cells to G-CIV. These ligands had no effect on adhesion of these cells to albumin-coated glass surfaces. Similar results were obtained for SRIA6 cells. These results parallel the results shown in Fig. 2 for human macrophages and confirm that SRs promote macrophage adhesion to G-CIV-coated surfaces. Furthermore, SRIIA4 and VB3 cells did not stain with monoclonal antibody IB4 by indirect immunofluorescence (not shown), suggesting that they do not express β₂ integrins. As expected, IB4 antibodies had no effect on the adhesion of SRIIA4 cells to G-CIV (Fig. 4).

The collagen-like domain of SRs is thought to contain this receptor's binding site for modified LDL (3, 24). To test whether peptides derived from this domain affect binding of SR-expressing U937 cells to G-CIV, we incubated G-CIV-coated slides with a synthetic peptide (SP1) corresponding to residues 327–343 of the collagen-like domain of bovine SRII, or with a control peptide (SP2), corresponding to residues 121–137 of the α helical domain of bovine SRII (3), for 10 min at room temperature, and then with SRIIA4 cells. SP1 inhibited adhesion of SRIIA4 cells
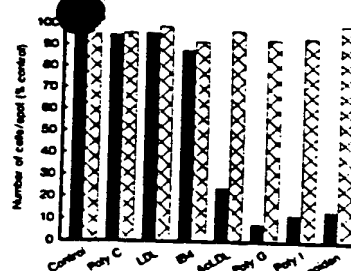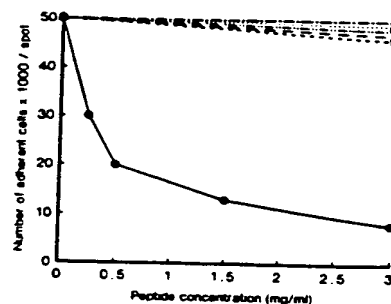

Fig. 5. **A synthetic peptide blocks adhesion of SRIIA4 to G-CIV.** Multispot glass slides were coated with 10 µg/spot glycated collagen IV or HSA as described under "Experimental Procedures." The indicated concentrations of synthetic peptide 1 (SP1) (corresponding to residues 326–343 of the collagen-like domain of SRII) or a control peptide (SP2) (corresponding to residues 121–137 of the α helical domain of SRII) or PLL were added in 10 µl/spot for 10 min at 37 °C. Then RPMI containing 1 mg/ml BSA and 5 × 10⁴ SRIIA4 cells was added per spot, and adhesion was allowed to proceed for 1 h at 37 °C as described under "Experimental Procedures." The number of adherent cells was quantitated using the neutral red assay. ●, SP1/G-CIV; ■, SP1/HSA; ▲, SP2/G-CIV; △, SP2/HSA; ○, PLL/G-CIV; □, PLL/HSA.

to G-CIV in a dose-dependent manner, whereas SP2 had no effect on cell adhesion (Fig. 5). Neither SP1 nor SP2 had any effect on adhesion of SRIIA4 cells to albumin-coated glass (Fig. 5). SP1 contains four positively and one negatively charged side chains (net positive charge of 3 at neutral pH) while SP2 has four negatively and one positively charged side chains (net negative charge of 3). It was possible that SP1 because of its net positive charge was nonspecifically masking the negative charges on G-CIV, thereby blocking binding sites for SRs. To test this possibility, we pretreated G-CIV-coated slides with poly-L-lysine (PLL) (M_r 5200, net positive charge 35) and then incubated them with SRIIA4 cells. PLL had no effect on binding of SRIIA4 cells to G-CIV (Fig. 5). Control experiments showed that PLL did not enhance significantly the adhesion of VB3 cells to G-CIV.

Adhesion of mononuclear phagocytes to surfaces bearing ligands for Fc or complement receptors leads to trapping of these receptors in the segment of the cells' plasma membrane that is adherent to the ligand-coated substrate, and to the disappearance of these receptors from the segments of the cells' plasma membrane not in contact with the substrate (25). The redistribution of surface receptors is assayed by the depletion of these receptors from the cells' "apical" membrane as measured by a reduced capacity of the cells to bind and/or endocytose particles
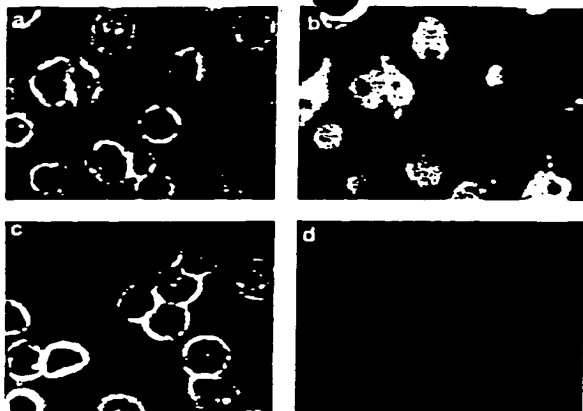
FIG. 6. **Redistribution of SRs in macrophages adhering to G-CIV.** 50 µl of RPMI medium containing 1 mg/ml BSA and 5 × 10⁴ monocyte-derived macrophages (7 days in culture) were allowed to adhere to uncoated or glycated collagen IV-coated Multispot slides for 1 h at 37 °C. Nonadherent cells were washed away, and RPMI containing 10 µg/ml DiI-labeled AcLDL was added to the cells. Uptake was allowed to proceed for 2 h, after which the cells were washed three times, fixed in 3.7% formaldehyde, and visualized using a Zeiss fluorescence microscope. Phase contrast (*a* and *c*) and fluorescence (*b* and *d*) micrographs of cells plated on glass (*a* and *b*) or on glycated collagen IV (*c* and *d*).

bearing ligands for these receptors from the medium (25). Indeed, macrophages plated on surfaces coated with G-CIV exhibited a marked reduction in uptake of DiI-AcLDL compared with cells plated on glass surfaces (Fig. 6) or on surfaces coated with IgG-containing immune complexes (not shown). On the other hand, macrophages plated on G-CIV showed no change in Fc receptor-mediated phagocytosis of IgG-coated sheep red blood cells compared to macrophages plated on glass (phagocytic indices: 906 and 948 red blood cells/100 macrophages, respectively). This result confirms that the reduced uptake of DiI-AcLDL by macrophages plated on G-CIV-coated surfaces reflects a selective effect of the substrate on SRs, not a general effect of the substrate on all endocytosis-promoting receptors.

SRs were originally identified by their ability to promote endocytosis of LDL whose apoprotein had been modified by acetylation or maleylation (1). Frazer *et al.* (5) have reported that SRs mediate the adhesion of mouse macrophages to serum-coated plastic surfaces, but they did not identify the ligand in the serum. We show here that G-CIV is an adhesion promoting ligand for SRs, and that a peptide from the putative ligand binding collagenous domain of SRs blocks adhesion of macrophages to G-CIV-coated surfaces. These findings suggest two mechanisms by which SRs may promote atherogenesis in diabetics. First, SRs promote monocyte adhesion to glucose-modi-

fied basement membrane proteins in the intima of arteries, thereby enhancing the accumulation/trapping of these cells at this site. Second, macrophages secrete proinflammatory cytokines and growth factors when they interact with SRs ligands and glycated proteins (14, 26). Interaction of macrophage SRs with glucose-modified extracellular matrix proteins, such as G-CIV, also may signal secretion of proinflammatory substances thereby stimulating the influx of monocytes and smooth muscle cells into the intima and enhancing the proliferation of smooth muscle cells.

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